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14. ABSTRACT The goal of this project is to develop biopsy based assays to assess the probability that patients with a negative biopsy or with a prostate cancer (CaP) Gleason score 6 (GS6) biopsy actually have "significant" CaP of Gleason score 7 or higher which was missed during the biopsy evaluations due to insufficient sampling. According to the DOD regulations, all research activities began after the approval of the research protocol by the HRPO at the end of March 2012. Since then, we have completed the transcriptomic profiling of the samples described in the original protocol. Furthermore, through additional funding and collaborations, we have significantly expanded the size and scope of the samples for genomic profiling. Transcriptomic profile (RNA-seq) of the expanded set of samples has been generated and mapped. We encountered some challenges in generating RRBS libraries from LCM samples which we overcame and we expect to have the RRBS data of the expanded set available shortly. Validation experiments were started by two new members of our team and are proceeding rapidly. Also, with the assistance of the biostatisticians in our group, case selection for all steps of the project is completed. Other research engagements funded separately, such as investigations of DNA-BPDE adducts and specific non-coding RNA in the benign tissues have promise in improving the accuracy of assays developed in this proposal. We are encouraged by our progress and are excited about the prospects of this research in improving the care of patients at risk of prostate cancer.

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Introduction:

Each year in the U.S. more than a million men with an elevated serum PSA or abnormal digital rectal exam undergo a prostate biopsy, and nearly 200,000 are found to have prostate cancer (CaP). Decisions to treat CaP are heavily influenced by the Gleason score (GS) of the tumor in the needle biopsy specimen. Gleason score is a measure of tumor differentiation based on the two most prevalent patterns of tumor growth. Patients whose entire tumor is composed of GS6 rarely progress, and recently, more men diagnosed with GS6 tumors on needle biopsy are selecting active surveillance rather than surgery or radiation therapy. In contrast, men with more poorly differentiated tumors (GS7 and higher) have a significantly increased risk of progression, and require treatment. Choosing the best treatment options for patients with biopsy GS6 is complicated by the fact that a biopsy procedure only samples a very small part of the prostate, and in about 30% of men, it underestimates the GS (Griffin, Yu et al. 2007). In those cases, men with GS7 and higher ($GS7^+$) prostate cancer are assumed to have GS6 tumors potentially leading to inappropriate treatment. In addition, because of the limited sampling and 30% false negative rate for detecting cancer (Stewart, Leibovich et al. 2001; Patel, Jones et al. 2004), many men with a negative biopsy result may have clinically significant prostate cancer. Because of that, many of the 800,000 patients with a negative biopsy undergo repeat biopsies which can be frustrating for both patients and urologists. When a pathologist examines a prostate needle biopsy specimen, the focus is on the identification of prostate cancer and appropriate Gleason scoring. Very little attention is paid to the "normal" areas which often comprise the majority of biopsy samples. This is despite a considerable body of evidence suggesting that molecular alterations associated with tumor in adjacent non-neoplastic cells, the so called "tumor field effect", can provide valuable clues regarding the status of the tumor. Remarkably, the field effect alterations have also been associated with aggressive prostate cancer (Malins, Gilman et al. 2004).

Body / Results:

The objective is to develop clinically relevant molecular models to predict significant prostate cancer with $GS7^+$ based on the prostate cancer field effect markers. This proposal will focus only on identification of significant tumors with $GS7^+$ because Gleason score is the single strongest predictor of outcome in men with prostate cancer, and has the greatest influence on the clinical management of men with prostate cancer. This proposal will concentrate on the "omics" areas where prostate cancer field effect has been best demonstrated, namely transcriptomic and epigenomics. There are two Aims. Aim I will identify and validate prostate cancer field effect markers associated with $GS7^+$ tumors. Aim II will develop and test molecular models for predicting upgrading in GS6 biopsies and for predicting $GS7^+$ cancer in a repeat biopsy.

Aim I will analyze 4 types of samples (Table I). These include non-cancerous tissues from CaP patients with (i) indolent GS6 CaP (N_{i6} = 5), (ii) GS3+4 CaP (N_{3+4} = 5), and (iii) GS 8 and higher CaP (N_{8+} = 5). We also analyze benign prostate tissues from patients free of CaP (BP = 5) as controls. BP

Table I: Bulk and LCM samples proposed in the application for the biomarker discovery step by the next generation sequencing

Sample	Bulk	LCM (HGPIN)
BP	5	
N_{i6}	5	5
N_{3+4}	5	5
N_{8+}	5	5
Total	**20**	**15**

samples are resected prostate tissues from patients who were not diagnosed with CaP but had their prostates resected in cystoprostatectomy operations because of bladder cancers. In the proposal for the first phase of the project, gene expression and epigenetic alterations are to be analyzed by next generation sequencing. Laser captured microdissection (LCM) is used to collect high grade prostatic intraepithelial neoplasia (HGPIN) lesions in 15 samples. The remaining samples are collected using bulk macro-dissection (Table I).

Research Accomplishments:

Collection of BP tissues and selection of cases for the next generation sequencing (NGS): Original plan called for collection of 15 HGPIN and 20 bulk samples of benign tissues for transcriptome and epigenetic profiling by NGS (Table I). However, subsequent investigations determined that it was crucial for the success of this project to increase the size and scope of samples (described under "Additional related research activities" below). We were able to secure additional funds from the Prostate SPORE and Center of Individualized Medicine (CIM) at the Mayo Clinic to expand the samples for genomic profiling without incurring additional cost to this DOD proposal. Additionally, we collaborated with Dr. Thibodeau who also holds a DOD grant for transcriptome sequencing of BP bulk samples. The expanded set includes tumor samples and associated epithelial cells collected by LCM as described in Table 2.

In coordination with the Tissue Request Acquisition Group (TRAG) at the Mayo Clinic, a process was implemented for the collection of resected prostates from cystoprostatectomy patients. Between samples collected since last year with the DOD protocol and ones collected under a previous IRB, we now have sufficient number of CPR for the validation studies.

Table 2: Bulk and LCM samples collected in this study for genomic profiling. Transcriptome profiling data (RNA-seq) for all these samples have been completed. Numbers in parenthesis indicate samples for which funds from this DOD proposal were used for generating the sequencing data.

	Bulk	**LCM**		
Sample	**benign Areas**	**HGPIN**	**aN**	**tumor**
BP	19 (0)			
N_{i6}	8 (8)	6 (6)	6 (0)	6 (0)
N_{3+4}	7 (7)	6 (6)	6 (0)	12 (0)
N_{8+}	12 (12)	5 (5)	5 (0)	5 (0)
Total	46 (27)	17 (17)	17 (0)	23 (0)

Collection of samples and DNA/RNA isolation: Cases with adequately large areas of HGPIN for LCM collections and benign areas that did not include any preneoplastic or tumor regions by macro-dissection in each of the N_{i6}, N_{3+4}, and N_{8+} categories were identified in our frozen prostate tissue registry. Additionally, we required that

sufficient quantities of tumor be available for separate LCM collection. All samples in this study were collected under IRB approved protocols.

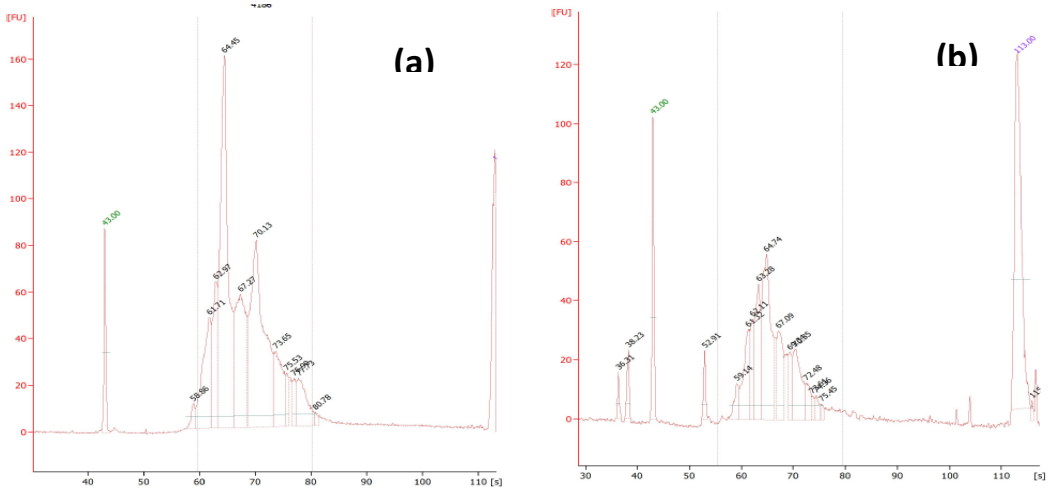
Under the supervision of Dr. Cheville, LCM samples have been collected by a meticulous and carefully implemented process to minimize degradation of nucleic acids, especially the RNA. Bulk areas for macro-dissection mostly from the same LCM cases were collected. Purified RNA from LCM samples by a Qiagen kit was of high quality (RIN numbers typically greater than 8) and quantity (tens of nano-grams). Also DNA from LCM samples by an Arturus kit was of sufficient quantity for the RRBS library prep (see below). DNA and RNA were simultaneously collected from the bulk samples by the AllPrep kit (Qiagen) and had very high quality and yield.

Library preparations for transcriptome profiling: Table2 describes samples that were submitted for transcriptome sequencing. We worked closely with the the Mayo Genomics Facility in preparing the libraries for RNA-seq. All LCM RNA samples used 5 nanograms starting material and produced very uniform output by NuGen protocol. Similarly, all bulk samples produced high quality TruSeq libraries.

Library preparations for epigenetics profiling: Sample requirements for methylation profiling by reduced representation bisulfite sequencing (RRBS) at the Genomics Facility included 100-500 ng starting material. This amount was beyond the yields of LCM experiments. We searched core facilities in other institutions and commercial sources for preparing RRBS libraries for our LCM samples, but did not identify a suitable option. We therefore proceeded to develop our own procedure for processing RRBS libraries from LCM samples. We examined or tested multiple protocols (Boyle, Clement et al. 2012; Smallwood and Kelsey 2012; Schillebeeckx, Schrade et al. 2013) including a commercial kit (BIOO Scientific). At the end, we were able to develop a robust protocol for both bulk and LCM samples (Figure 1). However, this step interjected a few months of delay in our scheduled plan.

Majority of the samples required for this study (Table1) are prepared and ready sequencing. Our immediate goal is to increase the number and types of samples as we have done for transcriptome analysis in Table 2.

Figure 1 Representative RRBS libraries from bulk (300 ng) and LCM samples (~15 ng). Observed peaks are characteristic of RRBS libraries in 180-400 bp range. Small peak at 52.9 is eliminated by gel purification.



Next generation sequencing and mapping of data: In order to enhance the chances of success, it is important to have high depth of sequencing coverage. That is because tumor associated genomic alterations in the benign tissue are often of lower magnitude than changes in the tumor. Therefore, as much as our budget allowed, we reduced multiplexing of samples per each lane of sequencing so the sequencing depth could be increased. Both LCM and bulk samples were multiplexed three per lane for transcriptome profiling. This amount of multiplexing allowed us to have close to 80 million fragments per sample which will be sufficient to identify small fold changes for most genes and also alternative splicing events which could provide valuable biomarkers for this project. We required RRBS libraries for the methylation sequencing to be multiplexed 4 per lane. If we find that a higher sequencing depth is needed for the epigenetics profiling, we will re-submit these libraries for additional sequencing.

Mapping of the transcriptome data including all samples described in Table 2 is completed by the Bioinformatics Core facility. Furthermore, mapping of the methylome data will be carried out by the same facility as soon as sequencing steps are completed.

Selection of markers from transcriptome and epigenetic markers: As described in the original application, data from our microarray data and RNA-seq data will be used to select the candidate transcript biomarkers. By analyzing our microarray data, we have selected two sets of candidates for examination in our RNA-seq data. These include non-coding RNA as well as protein coding transcripts. Two important non-coding RNA, MEG3 and TUG1, were down regulated compared to BP in both the benign tissue adjacent to cancer (BPC) and in prostate cancer. Interestingly, both of these genes can also be field effect biomarkers of significant prostate cancer. We are investigating the binding partners of these two promising RNA field effect biomarker genes in a separate funding proposal sponsored by the Mayo Prostate SPORE program (please see Additional related research activities). Other protein coding gene candidate genes selected are described in Table 3.

Table 3: Selected genes for examination in the RNA-Seq data; Transcripts with lower (upper panel) and higher (lower panel) expression in N_{8+}Compared with N_6 in our microarray data are selected based on the analysis of the microarray data. Important cancer genes are shown in bold.

Lower in N_{8+}Compared with N_6				
AHA1	CDH19	CENTB5	CLSTN2	CYP4F8
FABP4	GOPC	GREM1	HSD11B2	**IDS**
KIAA1618	LRRC31	PCDH20	PGC	PIP
TGM4	**TP53**	WT1		
Higher in N_{8+}Compared with N_6				
BMP5	DDX6	F2RL1	GDF15	HAS3
HFE	**JAK1**	**JUN**	KIAA1217	**MET**
NAV1	NPR3	NR5A2	OR51E2	PGF
PPM1E	RND1	SCGB1D2	SLC2A3	STC1
TFF3	TMEM37	**TOP2A**	**WNT4**	

Validation in FFPE surgical samples

Case selection: This step is completed. With the help of biostatisticians in our group, we have identified 90 indolent GS6, 60 GS3+4, and 70 GS8 and higher patients for which archival (FFPE) tissues are available. All these patients had their diagnostic biopsies done at the Mayo Clinic, so we will be able to verify differential expression of our biomarkers in the biopsy of these patients before moving into independent biopsy samples. Dr. Simone Terra, the new resident pathologist in our group has been examining tissue slides and selecting the right blocks for experiments.

Collection of HGPIN and benign areas and RNA and DNA isolation and QC inspection: Under the supervision of Dr. Cheville, HGPIN and areas of non-neoplastic FFPE tissues are being collected by Drs. Terra and Arvizo by using our automated LCM machine. Dr. Arvizo is a new member of our team and has been instrumental in moving the validation experiments forward. She joined our group in June of 2013 and was trained in handling biospecimens collected by LCM (see Additional related research activities). Dr. Arvizo examined several commercial sources for isolation of DNA and RNA from FFPE and selected the best performing kit by Qiagen. Together, Drs. Terra and Arvizo process about 5-6 cases per week including DNA/RNA isolation of the LCM samples and quality and quantity inspection.

Aim 1.c. and Aim 2 biopsy Case selection: Under IRB guidance we identified the prostate cancer biopsies that are needed for this study in the Mayo Clinic archives. Seven types of biopsy samples will be acquired.

1. Last biopsies from patients with multiple negative biopsies; Aims Ic (n = 50) and IIb (n=70). Our search identified 2308 patients who have had at least 2 biopsies at the Mayo Clinic.
2. Negative cores of Gleason score 6 (GS6) biopsies from patients identified with insignificant GS6 tumors after surgeries; Aim Ic (n = 100). Our search identified 5460 patients with a GS6 biopsy.
3. Negative cores of GS3+4 biopsies; Aim Ic (n = 50). Our search identified 1519 patients with GS3+4 biopsies.
4. Negative cores of GS8 and higher biopsies; Aim Ic (n = 50). Our search identified 763 patients with GS8 and higher biopsies.
5. Negative cores of Gleason score 6 (GS6) biopsies; Aim IIa (n = 700). Our search identified 5460 patients with a GS6 biopsy. Within all these specimens and data, from 2006-2007, a consecutive prostate needle biopsy and RP specimen Mayo database has been created for GS 6 tumors. This is comprised of 356 with an upgrading (GS>7) rate of 21% at RP. Upgrading occurs when the needle biopsy specimen is GS6 (and patient would be candidate for active surveillance) but RP specimen is GS7 or higher (indicating needle biopsy specimen sampling error). A separate more recent biopsy group is identified once again with paired biopsy and RP specimens at Mayo from 2010-2012 for

validation work. This consists of 187 with an upgrading rate of 37%. Therefore 543 (356+187) patient biopsies have been characterized. We will characterize the remaining cases needed to reach 700.

6. Penultimate negative biopsies from patients identified with a GS6 cancer on the last biopsy; Aim IIb (n = 70). Our search identified 674 such patients.
7. Penultimate negative biopsies from patients identified with a GS7 and higher cancer on the last biopsy; Aim IIb (n = 70). Our search identified 232 patients. In this group, 72 patients would be 85 years or older by the second year of the study based on their age at their first visit to the Mayo Clinic.

Additional related research activities: In addition to the steps described above, we have engaged in other research activities which will enhance our abilities to accomplish PC100553 goals. These include:

Addition of two new members to the group: Simone B. Terra, M.D. is a very diligent and excellent pathologist who is carefully going through the patients slides and selecting the right tissue blocks and biopsy cores for this project. Her contributions are invaluable to this research.

Dr. Rochelle Arvizo joined our group in June 2013 to take over the experimental aspects of this project. Dr. Arvizo has an impressive background in scientific research including 4 years of relevant experience. She has first author publications in high impact factor (IF) journals, such as Chemical Society reviews (IF = 24.9), Nano Letters (IF = 13.0), and Proceedings of the National Academy of Sciences (IF = 9.7). Since she joined our group, she has been trained in LCM procedures and has been working closely with Dr. Terra in processing the validation cases. She is also in charge of the DNA/RNA isolation from archival FFPE tissues collected by LCM and from biopsy cores and biomarker validation experiments by RT-PCR and methylation specific PCR.

Additional funding from the Mayo Prostate SPORE: In February of 2013, we secured developmental funding from the Mayo Prostate SPORE. Our proposal included two Aims. The first Aim was to supplement our DOD proposal (PC100553) for genomic profiling. This aim included RNA-seq and methylation profiling of the associated normal epithelial cells collected by LCM. The DOD proposal provides genomic profiling of two types of samples including; (a) high grade PIN (HGPIN) collected by LCM; and (b) bulk normal areas which include a mixture of epithelial and endothelial cells along with stroma. Genomic profiling that includes stroma is important because the role of stroma in prostate cancer development has been established in numerous reports. However, since CaP arises in the epithelial cells, it is important to profile pure population of epithelial cells so that genomic signals that give rise to cancer can be enriched. In all cases where we collected HGPIN, we also collected the associated normal (aN) epithelial cells. Transcriptome (RNA-seq) data from aN cells (Table 2) were generated using these funds and we plan to generate epigenetics profile by RRBS of the aN samples in the near future.

Aim2 of the Developmental award is also completely in line with the goals of our DOD proposal. This aim finds the binding partners of non-coding RNAs TUG1 and MEG3 and their ability as field effect biomarkers of "significant" CaP. These two long non-coding RNAs are significantly down-regulated compared with BP in

BPC and CaP (Figure 2). Importantly, these RNA genes or their binding partners are also "field effect" biomarkers of significant prostate cancer and therefore will be tested in our validation experiments.

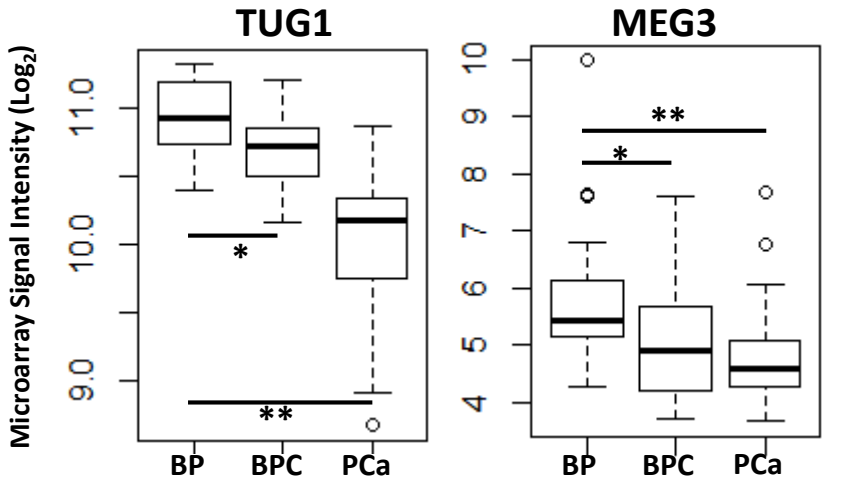


Figure 2: MEG3 and TUG1 are significantly down regulated in BPC and CaP compared with BP. Box plots are microarray data from BP (n = 28), BPC (n = 36), and CaP (PCa, n = 37). For most probesets, this down-regulation was significant even after adjusting for multiple comparisons (q-value < 0.001). * and ** represent p-values ≤ 0.008 and ≤ 0.0008, respectively.

Collaborations with the Mayo Center of Individualized Medicine (CIM): Identification of cancer "field effect" biomarkers by genome profiling approaches are challenging. Unlike cancer biomarkers that typically have many fold differential expression in "cancer versus normal" comparisons, cancer "field effect" biomarkers are subtle and difficult to distinguish from unrelated small changes in genomic profiling. Our paper, which was published last year, demonstrated that using genomic alterations in prostate cancer as a guide for selecting "field effect" biomarkers can significantly increase chances of reducing false positive and identifying markers that validate in independent experiments. Therefore, we sought to generate genomic profile of tumor samples that would guide our biomarker selection in our DOD study. We established collaboration with Dr. Jeffrey Karnes who is interested in identifying indolent cancers based on the genomic rearrangements (such as chromosomal translocations or deletions, gene fusions, etc.) in tumor cells. Through this collaboration (which is funded by the Mayo CIM) in addition to the HGPIN samples for the PC100553 project, we have been collecting tumor cells from indolent GS6 and from GS7 and higher tumors by LCM. The RNA-Seq profiles generated in LCM tumor samples in Table 2 was funded by this collaboration with the CIM. We are also planning to generate methylation profiles of these samples by RRBS in the near future.

BPDE-DNA adducts as "field effect" biomarkers of "significant" prostate cancer: Previous studies by immunohistochemistry (IHC) have shown that the concentrations of BPDE-DNA adducts in the benign glands in prostate tissues that contain prostate cancer are correlated with the grade of prostate cancer ($p = 0.008$)(Rybicki, Rundle et al. 2004). Prostates that contain high grade of CaP had higher concentration of BPDE-DNA adducts in their benign glands compared with prostates that had low grades of CaP. Therefore, these adducts can serve as "field effect" biomarkers of "significant" prostate cancer. We obtained funding through NIH R21 mechanism to develop very sensitive protocols for measuring these adducts in human tissues including prostates. We examine if the genomic DNA adducts in the benign glands of prostate will add to the accuracy of final models in predicting significant CaP.

Collaboration with Dr. Thibodeau: We found out that Dr. Thibodeau at the Mayo Clinic also has a DOD award which requires transcriptome profiling of BP. To avoid duplication of efforts and to save funds, we shared our BP biospecimens with Dr. Thibodeau's group which was used to generate the RNA-seq data from 19 BP samples in Table1.

Publications and presentations: We published our previous results related to the CaP field effect in an article in the American Journal of Pathology (Kosari, Cheville et al. 2012). We presented these results in the AACR annual meeting in April 2012 with the acknowledgement of the DOD sponsorship. Additionally, our investigations of genomic rearrangements and chromosomal abnormalities identified strong evidence for prostate cancer field effects. Some of these data were published in 2012 (Murphy, Cheville et al. 2012).

Reportable Outcomes

We have generated libraries for transcriptome sequencing of all samples that were planned for this project. Furthermore, based on this DOD proposal, we were able to secure developmental funding through the Prostate SPORE program. This proposal also aided in securing collaborations with the CIM and Dr. Thibodeau. Through these activities, we were able to considerably expand the size and the scope of the sample sets. This expansion will be a very important step in improving the chances for success in this project. Based on our previously reported results, "Field Effect" genomic changes are more subtle than one can expect from "cancer versus normal" comparisons. Therefore having a sufficient number of samples and including tumor genomic profile to guide selection of FE biomarkers will be crucial.

We have reported on DNA rearrangements (such as translocations and deletions) in the CaP adjacent non-neoplastic cells in 2012 (Murphy, Cheville et al. 2012). Another publication (Kosari, Cheville et al. 2012) describes our previous work related to the CaP field effect. With the acknowledged support from this grant, this work was presented in the AACR meeting in April 2012.

Conclusions:

We have made significant progress in the last year towards our goals by completing transcriptomic profiling and especially by expanding the scope and the size of samples, mostly through additional funding and new collaborations. In generating the epigenomic profiling, we encountered some challenges in that we had to develop expertise in generating RRBS libraries for the LCM samples. This step delayed our progress, but we were able to overcome this roadblock and soon we will be able to have the methylation sequencing data.

Because the project started with some delay in 2012 after the HRPO approval of the research protocol by the DOD and because of the un-anticipated challenge of generating the RRBS for the LCM samples which took our focus away from the validation experiments, some of the allocated budget for the year 2 including reagents for experiments is un-spent. However, with the addition of two new members to the team and our current momentum, we expect these funds will soon be needed to complete the planned experiments. In retrospect,

we realize that our schedule for the initial year of the project was overloaded compared with the later years. With most of the experiments related to the initial phase completed, we are looking forward to the results of our validation and model building/testing steps. These remaining experiments are mostly PCR based and we don't expect major challenges. We are very excited about the prospects of this project and believe that it has significant translational potential for the improving the care of patients who are at risk of prostate cancer

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